Impaired Cholesterol Efflux Capacity of High-Density Lipoprotein Isolated From Interstitial Fluid in Type 2 Diabetes Mellitus-Brief Report

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Impaired Cholesterol Efflux Capacity of High-Density Lipoprotein Isolated From Interstitial Fluid in Type 2 Diabetes Mellitus—Brief Report

Johanna Apro, Uwe J.F. Tietge, Arne Dikkers, Paolo Parini, Bo Angelin, Mats Rudling

Objective—Patients with type 2 diabetes mellitus (T2D) have an increased risk of cardiovascular disease, the mechanism of which is incompletely understood. Their high-density lipoprotein (HDL) particles in plasma have been reported to have impaired cholesterol efflux capacity. However, the efflux capacity of HDL from interstitial fluid (IF), the starting point for reverse cholesterol transport, has not been studied. We here investigated the cholesterol efflux capacity of HDL from IF and plasma from T2D patients and healthy controls.

Approach and Results—HDL was isolated from IF and peripheral plasma from 35 T2D patients and 35 age- and sex-matched healthy controls. Cholesterol efflux to HDL was determined in vitro, normalized for HDL cholesterol, using cholesterol-loaded macrophages. Efflux capacity of plasma HDL was 10% lower in T2D patients than in healthy controls, in line with previous observations. This difference was much more pronounced for HDL from IF, where efflux capacity was reduced by 28% in T2D. Somewhat surprisingly, the efflux capacity of HDL from IF was lower than that of plasma HDL, by 15% and 32% in controls and T2D patients, respectively.

Conclusion—These data demonstrate that (1) HDL from IF has a lower cholesterol efflux capacity than plasma HDL and (2) the efflux capacity of HDL from IF is severely impaired in T2D when compared with controls. Because IF comprises the compartment where reverse cholesterol transport is initiated, the marked reduction in cholesterol efflux capacity of IF-HDL from T2D patients may play an important role for their increased risk to develop atherosclerosis. **(*Arterioscler Thromb Vasc Biol*. 2016;36:787-791. DOI: 10.1161/ATVBAHA.116.307385.)**

Key Words: diabetes mellitus, type 2 ■ extracellular fluid ■ high-density lipoprotein cholesterol ■ low-density lipoprotein cholesterol ■ macrophages

Patients with type 2 diabetes (T2D) display an increased risk for premature cardiovascular disease and death.[1] Several factors may contribute to this, including hyperglycemia, dyslipidemia, and inflammation.[1] Levels of plasma low-density lipoprotein (LDL) cholesterol are usually not markedly elevated in T2D patients; instead they often present with reduced high-density lipoprotein (HDL) cholesterol levels. Importantly, lipid-lowering therapy is of clear benefit for T2D patients.[2]

We recently investigated the hypothesis that T2D patients might have increased levels of LDL in interstitial fluid (IF) because of an enhanced leakage over the vascular wall.[3] However, we unexpectedly observed the opposite, ie, that T2D patients have less apolipoprotein B (apoB)–containing lipoproteins (very low-density lipoprotein and LDL) in IF relative to serum compared with healthy controls.[3] These results indicated an increased accumulation or catabolism of apoB-containing particles in the interstitial compartment in T2D. In contrast, the level of HDL cholesterol in IF did not differ between T2D patients and healthy controls.[3]

HDL metabolism plays an important role for the development of atherosclerosis, and the inverse relationship between HDL cholesterol levels and cardiovascular disease is well established.[4] However, attempts to pharmacologically increase plasma HDL-cholesterol failed to show clinical benefit.[5–7] It has recently become evident that the functional properties of HDL particles are important for their atheroprotective function, more than the circulating levels per se.[8] One major function of HDL is its key role in reverse cholesterol transport, ie, transport of cholesterol from peripheral tissues to the liver for subsequent biliary and fecal excretion. In this process, the capacity of the HDL particle to promote cholesterol

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Nonstandard Abbreviations and Acronyms	
FPLC	fast performance liquid chromatography
IF	interstitial fluid
T2D	type 2 diabetes mellitus

efflux from peripheral cells is considered a crucial step.[9] Thus, measurements of this efflux capacity have shown to be independently related to the risk for cardiovascular events.[10] Accordingly, T2D patients generally have been demonstrated to display reduced cholesterol efflux capacity.[11–14]

As reverse cholesterol transport is initiated in the extravascular compartment in peripheral tissues,[9] evaluating efflux capacity of HDL from IF might be more relevant than studying plasma HDL. Various aspects of HDL metabolism have previously been studied in IF (ie, peripheral lymph).[15–17] However, to the best of our knowledge, there are no reports on HDL-mediated cholesterol efflux using HDL from IF, neither in healthy subjects nor in T2D patients. Therefore, we measured in vitro cholesterol efflux capacity of HDL isolated from both IF and plasma obtained from a previous study of T2D patients and healthy controls.[3]

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Lipoprotein Sizes in Serum and IF From T2D Patients and Healthy Controls

LDL and HDL particle diameters, calculated from the fast performance liquid chromatography (FPLC) retention times, are shown in the Table (absolute retention times are given in Table I in the online-only Data Supplement and the FPLC profiles are given in Figure IA and IB in the online-only Data Supplement). Compared with controls, the calculated HDL particle diameters in T2D patients were reduced by 6.3% and 4.6% in serum and IF, respectively. When comparing sizes of HDL particles in IF with those in serum in the same study groups, we found that the HDL particle size in IF was increased by 4.4% in controls and by 6.4% in T2D patients (Table).

The LDL particle size in T2D patients was smaller than in controls, by 2.7% and 1.9% in serum and IF, respectively. LDL size did not differ between serum and IF, neither in T2D patients nor in controls (Table).

Lipid Composition of HDL in Serum and IF From T2D Patients and Healthy Controls

Phospholipid content of HDL from serum and IF was analyzed by FPLC (the FPLC profiles are shown in Figure IIC and IID in the online-only Data Supplement). T2D patients were found to have reduced levels of phospholipids in HDL from both serum and IF when compared with controls (Table II in the online-only Data Supplement). As described previously,[3] triglyceride and unesterified cholesterol contents of HDL, together with esterified cholesterol content of HDL (calculated from total cholesterol and unesterified cholesterol) were determined and are shown in Table II in the online-only Data Supplement; the FPLC profiles for unesterified cholesterol and triglycerides are given in Figures IC and ID and IIA and IIB. The percentage composition of HDL lipids in relation to total (Table) was calculated from the lipid data in Table II in the online-only Data Supplement. From these calculations, it was found that serum HDL from T2D patients had decreased content of unesterified cholesterol and increased triglyceride content compared with healthy controls, whereas for esterified cholesterol and phospholipids, there were no differences (Table). HDL from IF from T2D patients had a reduced content of esterified cholesterol and an increased content of phospholipids. For unesterified cholesterol and triglycerides, there were no differences (Table). When comparing lipid composition in HDL from serum and IF, respectively, it was found that HDL from IF contained more esterified cholesterol, unesterified cholesterol, and triglycerides, but substantially less phospholipids than did HDL from serum, in both T2D patients and healthy controls (Table).

HDL-Mediated Cholesterol Efflux Capacity in Plasma and IF in T2D Patients and Healthy Controls

Cholesterol efflux to HDL, normalized for HDL cholesterol, was assayed using a standardized in vitro system based on THP-1–derived macrophage foam cells loaded with 3H-cholesterol.[18] As expected, plasma HDL from T2D patients had a 10% lower efflux capacity when compared with healthy controls (Figure). The efflux capacity of IF-HDL was reduced by 28% in samples from T2D patients compared with those from controls (Figure). Comparing the efflux capacities of HDL from IF with those from plasma in controls and in T2D, respectively, it was found that efflux to HDL-IF was 15% lower in healthy controls whereas the reduction in T2D was even more pronounced (32%; Figure).

Discussion

To the best of our knowledge, cholesterol efflux measurements using HDL isolated from IF have not been reported previously. In our study, we could demonstrate that cholesterol efflux to HDL of interstitial origin was clearly of lower magnitude than that to plasma HDL, in both healthy controls and T2D patients. Because the process of cholesterol loading of HDL particles is thought to mainly take place in peripheral tissues,[16,17] the evaluation of cholesterol efflux capacity of HDL from IF may, therefore, be of higher physiological relevance than measurements of the efflux capacity of HDL from plasma.

We could confirm the results of previous reports by showing a 10% reduction in plasma HDL cholesterol efflux in T2D patients in comparison with healthy subjects.[11–14] However, not all studies have been able to detect differences between T2D patients and healthy controls,[19,20] and 1 study has reported an increased efflux capacity in T2D patients.[21] These incongruent results might be related to not only the varying degree of severity of disease of the patients studied but also differences in the cell systems used in the efflux assays. It is important to

Table. LDL and HDL Sizes and Lipid Composition of HDL in Serum and IF From T2D Patients and Controls

	Serum	*P* Value (Healthy Controls vs T2D Patients)	IF	*P* Value (Healthy Controls vs T2D Patients)	*P* Value (Serum vs IF)
Healthy controls					
LDL (nm)	21.11±0.34	…	21.07±0.40	…	n.s.
HDL (nm)	8.91±0.35	…	9.31±0.46	…	<0.01
CE in HDL (%)	28.3±4.2	…	41.5±5.0	…	<0.0001
FC in HDL (%)	10.3±2.2	…	17.9±2.8	…	<0.0001
TG in HDL (%)	4.9±2.3	…	8.5±2.8	…	<0.0001
PL in HDL (%)	56.5±4.6	…	32.1±4.6	…	<0.0001
T2D patients					
LDL (nm)	20.53±0.45	<0.001	20.68±0.37	<0.001	n.s.
HDL (nm)	8.35±0.40	<0.001	8.88±0.66	<0.001	<0.001
CE in HDL (%)	27.3±7.1	n.s.	35.4±6.5	<0.001	<0.0001
FC in HDL (%)	8.8±3.1	<0.01	16.8±4.3	n.s.	<0.0001
TG in HDL (%)	6.9±2.1	<0.0001	9.8±3.7	n.s.	<0.001
PL in HDL (%)	57.0±7.7	n.s.	38.0±4.4	<0.0001	<0.0001

Data are mean±SD. For percentage lipid composition, serum data were missing from 3 healthy controls and IF data from 1 healthy control. *P* values indicate the significance of difference from the same fluid in the control group or between serum and IF within the individual groups, as indicated (2-way ANOVA followed by Bonferroni multiple comparison test). CE indicates esterified cholesterol; FC, unesterified cholesterol; HDL, high-density lipoprotein; IF, interstitial fluid; LDL, low-density lipoprotein; PL, phospholipid; T2D, type 2 diabetes mellitus; and TG, triglyceride.

note that the reduced capacity for cholesterol efflux that we observed for HDL from plasma of T2D patients was much more pronounced for HDL from IF.

A potential limitation of our study is the fact that some T2D patients were on statin and fibrate treatments, alone or in combination, a situation that might have improved cholesterol efflux.[18,22,23] However, also in this case, the published literature is not consistent because unaltered[21,23,24] or even decreased[24] efflux capacity has been observed. We did not see any significant difference comparing T2D patients with or without lipid-lowering therapy in this respect (Table III in the online-only Data Supplement). The fact that many patients had documented cardiovascular disease should also be noted.

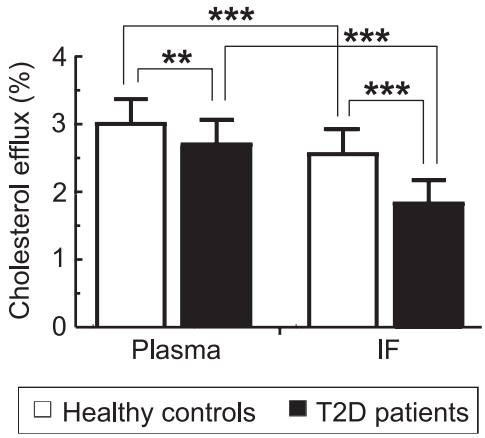

Figure. Cholesterol efflux capacity of high-density lipoprotein from plasma and interstitial fluid (IF) from type 2 diabetes mellitus (T2D) patients and healthy controls. Data are presented as mean±SD. Plasma was available from all subjects, whereas IF was available from 21 healthy controls and 24 T2D patients. ***P*<0.01, ****P*<0.001 (2-way ANOVA followed by Bonferroni multiple comparison test).

Small HDL particles have been reported to be more efficient to promote cholesterol efflux from cells.[25,26] Our finding of reduced HDL particle size in IF and serum from T2D patients may at first appear somewhat puzzling although the observation of a reduced size of serum HDL in T2D is in agreement with previous work.[27] It should be pointed out that the analysis of different subclasses of HDL is not possible using our technique. Increased glycation of HDL in T2D may contribute to the reduced cholesterol efflux[28] although such a phenomenon has also not been found universally.[21] From our calculated data on percentage composition of the different lipids in HDL, we show that the percentage of esterified cholesterol in serum HDL, the main component of the core of HDL, did not differ between healthy controls and T2D patients. For HDL from IF, the percentage of esterified cholesterol was reduced in T2D patients, which could contribute to the reduced HDL size.

Previous work has concluded that HDL from peripheral lymph carries more cholesterol than can be explained by transendothelial transfer of HDL from plasma,[16] and that the infusion of apoAI/phosphatidylcholine discs increases the cholesterol content of HDL in peripheral lymph.[17] Those results support the hypothesis that HDL acquires cholesterol within peripheral tissues. From this reasoning, we expected that the cholesterol efflux capacity should be higher for HDL isolated from IF than from plasma. Instead, we found the opposite. The reduced cholesterol efflux capacity of HDL from IF compared with those from plasma was accompanied by increased HDL size, in both patients and controls. This relationship fits well with the previously reported association between small HDL and increased efflux capacity.[25,26]

The phospholipid content of HDL is shown to be one of the major drivers of cholesterol efflux.[29] The reduced cholesterol efflux to IF-HDL may, therefore, at least in part, be explained by the substantially reduced phospholipid content in those particles compared with serum HDL. These results further support the importance of HDL-phospholipid for cholesterol efflux capacity.

Interestingly, it has been shown in vitro that phospholipid hydrolysis by endothelial lipase contributes to transendothelial transport of HDL.[30] This may very well explain the reduced level of HDL-phospholipid in IF compared with serum. This transendothelial movement of HDL also reduces HDL size.[30] In this study, we found increased HDL size in IF, which may be explained by increased percentage of esterified cholesterol. Although not completely comparable, our results showing larger HDL size in suction blister fluid are also in agreement with the reported increased abundance of large HDL particles in peripheral prenodal lymph.[16] As HDL particles are proposed to acquire cholesterol in IF before returning to plasma via the lymph, it is reasonable that HDL size is increased in lymph. HDL isolated from suction blister fluid may already have acquired saturating levels of cholesterol from peripheral cells, resulting in larger size and decreased detected efflux capacity.

Analyzing samples from the same patients and controls, we recently showed that T2D patients have unexpectedly reduced levels of apoB-containing lipoproteins in IF, possibly mirroring an increased uptake by, or adhesion to, interstitial constituents. In contrast, the IF:serum ratio for HDL was not influenced by T2D.[3] The present demonstration of a significantly lower cholesterol efflux to HDL from IF suggests that, in addition to an increased peripheral disposal of apoB-containing lipoproteins, the capacity to remove deposited cholesterol is reduced in T2D. Altogether, our results indicate that the interstitial compartment, which should reflect the metabolic conditions in the arterial wall, may be an important location for several disturbances of cholesterol metabolism that might explain the increased propensity for atherosclerosis in T2D.

In conclusion, the capacity to promote cholesterol efflux is considerably lower for HDL from IF than HDL from plasma, both in healthy controls and T2D patients. Moreover, in IF from T2D patients, the efflux capacity of HDL is strongly impaired when compared with that in healthy controls. Interventions directed at promoting HDL-mediated cholesterol efflux in the IF compartment may be a successful new way to target the increased risk of atherosclerosis and cardiovascular disease in T2D.

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Disclosures

None.

References

1. Mazzone T, Chait A, Plutzky J. Cardiovascular disease risk in type 2 diabetes mellitus: insights from mechanistic studies. *Lancet*. 2008;371:1800–1809. doi: 10.1016/S0140-6736(08)60768-0.
2. Collins R, Armitage J, Parish S, Sleigh P, Peto R; Heart Protection Study Collaborative Group. MRC/BHF Heart Protection Study of cholesterol-lowering with simvastatin in 5963 people with diabetes: a randomised placebo-controlled trial. *Lancet*. 2003;361:2005–2016.
3. Apro J, Parini P, Broijersén A, Angelin B, Rudling M. Levels of atherogenic lipoproteins are unexpectedly reduced in interstitial fluid from type 2 diabetes patients. *J Lipid Res*. 2015;56:1633–1639. doi: 10.1194/jlr.P058842.
4. Sharrett AR, Ballantyne CM, Coady SA, Heiss G, Sorlie PD, Catellier D, Patsch W; Atherosclerosis Risk in Communities Study Group. Coronary heart disease prediction from lipoprotein cholesterol levels, triglycerides, lipoprotein(a), apolipoproteins A-I and B, and HDL density subfractions: The Atherosclerosis Risk in Communities (ARIC) Study. *Circulation*. 2001;104:1108–1113.
5. Landray MJ, Haynes R, Hopewell JC, Parish S, Aung T, Tomson J, Wallendszus K, Craig M, Jiang L, Collins R, Armitage J. Effects of extended-release niacin with laropiprant in high-risk patients. *N Engl J Med*. 2014;371:203–212
6. Boden WE, Probstfield JL, Anderson T, Chaitman BR, Desvignes-Nickens P, Koprowicz K, McBride R, Teo K, Weintraub W. Niacin in patients with low hdl cholesterol levels receiving intensive statin therapy. *N Engl J Med*. 2011;365:2255–2267
7. Schwartz GG, Olsson AG, Abt M, et al; dal-OUTCOMES Investigators. Effects of dalcetrapib in patients with a recent acute coronary syndrome. *N Engl J Med*. 2012;367:2089–2099. doi: 10.1056/NEJMoa1206797.
8. Triolo M, Annema W, Dullaart RP, Tietge UJ. Assessing the functional properties of high-density lipoproteins: an emerging concept in cardiovascular research. *Biomark Med*. 2013;7:457–472. doi: 10.2217/bmm.13.35.
9. Rosenson RS, Brewer HB Jr, Davidson WS, Fayad ZA, Fuster V, Goldstein J, Hellerstein M, Jiang XC, Phillips MC, Rader DJ, Remaley AT, Rothblat GH, Tall AR, Yvan-Charvet L. Cholesterol efflux and atheroprotection: advancing the concept of reverse cholesterol transport. *Circulation*. 2012;125:1905–1919. doi: 10.1161/CIRCULATIONAHA.111.066589.
10. Rohatgi A, Khera A, Berry JD, Givens EG, Ayers CR, Wedin KE, Neeland IJ, Yuhanna IS, Rader DR, de Lemos JA, Shaul PW. HDL cholesterol efflux capacity and incident cardiovascular events. *N Engl J Med*. 2014;371:2383–2393. doi: 10.1056/NEJMoa1409065.
11. Syvänne M, Castro G, Dengremont C, De Geitere C, Jauhiainen M, Ehnholm C, Michelagnoli S, Franceschini G, Kahri J, Taskinen MR. Cholesterol efflux from Fu5AH hepatoma cells induced by plasma of subjects with or without coronary artery disease and non-insulin-dependent diabetes: importance of LpA-I:A-II particles and phospholipid transfer protein. *Atherosclerosis*. 1996;127:245–253.
12. Autran D, Attia N, Dedecjus M, Durlach V, Girard-Globa A. Postprandial reverse cholesterol transport in type 2 diabetic patients: effect of a lipid lowering treatment. *Atherosclerosis*. 2000;153:453–460.
13. Attia N, Nakbi A, Smaoui M, Chaaba R, Moulin P, Hammami S, Hamda KB, Chanussot F, Hammami M. Increased phospholipid transfer protein activity associated with the impaired cellular cholesterol efflux in type 2 diabetic subjects with coronary artery disease. *Tohoku J Exp Med*. 2007;213:129–137.
14. Zhou H, Tan KC, Shiu SW, Wong Y. Determinants of leukocyte adenosine triphosphate-binding cassette transporter G1 gene expression in type 2 diabetes mellitus. *Metabolism*. 2008;57:1135–1140. doi: 10.1016/j.metabol.2008.03.020.
15. Miller NE, Olszewski WL, Hattori H, Miller IP, Kujiraoka T, Oka T, Iwasaki T, Nanjee MN. Lipoprotein remodeling generates lipid-poor apolipoprotein A-I particles in human interstitial fluid. *Am J Physiol Endocrinol Metab*. 2013;304:E321–E328. doi: 10.1152/ajpendo.00324.2012.
16. Nanjee MN, Cooke CJ, Wong JS, Hamilton RL, Olszewski WL, Miller NE. Composition and ultrastructure of size subclasses of normal human

peripheral lymph lipoproteins: quantification of cholesterol uptake by HDL in tissue fluids. *J Lipid Res*. 2001;42:639–648.
17. Nanjee MN, Cooke CJ, Garvin R, Semeria F, Lewis G, Olszewski WL, Miller NE. Intravenous apoA-I/lecithin discs increase pre-beta-HDL concentration in tissue fluid and stimulate reverse cholesterol transport in humans. *J Lipid Res*. 2001;42:1586–1593.
18. Triolo M, Annema W, de Boer JF, Tietge UJ, Dullaart RP. Simvastatin and bezafibrate increase cholesterol efflux in men with type 2 diabetes. *Eur J Clin Invest*. 2014;44:240–248. doi: 10.1111/eci.12226.
19. Dullaart RP, Annema W, de Boer JF, Tietge UJ. Pancreatic β-cell function relates positively to HDL functionality in well-controlled type 2 diabetes mellitus. *Atherosclerosis*. 2012;222:567–573. doi: 10.1016/j.atherosclerosis.2012.03.037.
20. Dullaart RP, van Tol A. Twenty four hour insulin infusion impairs the ability of plasma from healthy subjects and Type 2 diabetic patients to promote cellular cholesterol efflux. *Atherosclerosis*. 2001;157:49–56.
21. Low H, Hoang A, Forbes J, Thomas M, Lyons JG, Nestel P, Bach LA, Sviridov D. Advanced glycation end-products (AGEs) and functionality of reverse cholesterol transport in patients with type 2 diabetes and in mouse models. *Diabetologia*. 2012;55:2513–2521. doi: 10.1007/s00125-012-2570-9.
22. Guerin M, Egger P, Soudant C, Le Goff W, van Tol A, Dupuis R, Chapman MJ. Dose-dependent action of atorvastatin in type IIB hyperlipidemia: preferential and progressive reduction of atherogenic apoB-containing lipoprotein subclasses (VLDL-2, IDL, small dense LDL) and stimulation of cellular cholesterol efflux. *Atherosclerosis*. 2002;163:287–296.
23. Franceschini G, Calabresi L, Colombo C, Favari E, Bernini F, Sirtori CR. Effects of fenofibrate and simvastatin on HDL-related biomarkers in low-HDL patients. *Atherosclerosis*. 2007;195:385–391. doi: 10.1016/j.atherosclerosis.2006.10.017.
24. Sviridov D, Hoang A, Ooi E, Watts G, Barrett PH, Nestel P. Indices of reverse cholesterol transport in subjects with metabolic syndrome after treatment with rosuvastatin. *Atherosclerosis*. 2008;197:732–739. doi: 10.1016/j.atherosclerosis.2007.07.007.
25. Camont L, Lhomme M, Rached F, Le Goff W, Nègre-Salvayre A, Salvayre R, Calzada C, Lagarde M, Chapman MJ, Kontush A. Small, dense high-density lipoprotein-3 particles are enriched in negatively charged phospholipids: relevance to cellular cholesterol efflux, antioxidative, antithrombotic, anti-inflammatory, and antiapoptotic functionalities. *Arterioscler Thromb Vasc Biol*. 2013;33:2715–2723. doi: 10.1161/ATVBAHA.113.301468.
26. Du XM, Kim MJ, Hou L, Le Goff W, Chapman MJ, Van Eck M, Curtiss LK, Burnett JR, Cartland SP, Quinn CM, Kockx M, Kontush A, Rye KA, Kritharides L, Jessup W. HDL particle size is a critical determinant of ABCA1-mediated macrophage cellular cholesterol export. *Circ Res*. 2015;116:1133–1142. doi: 10.1161/CIRCRESAHA.116.305485.
27. Taskinen MR. Diabetic dyslipidaemia: from basic research to clinical practice. *Diabetologia*. 2003;46:733–749. doi: 10.1007/s00125-003-1111-y.
28. Duell PB, Oram JF, Bierman EL. Nonenzymatic glycosylation of HDL and impaired HDL-receptor-mediated cholesterol efflux. *Diabetes*. 1991;40:377–384.
29. Fournier N, Paul JL, Atger V, Cogny A, Soni T, de la Llera-Moya M, Rothblat G, Moatti N. HDL phospholipid content and composition as a major factor determining cholesterol efflux capacity from Fu5AH cells to human serum. *Arterioscler Thromb Vasc Biol*. 1997;17:2685–2691.
30. Robert J, Lehner M, Frank S, Perisa D, von Eckardstein A, Rohrer L. Interleukin 6 stimulates endothelial binding and transport of high-density lipoprotein through induction of endothelial lipase. *Arterioscler Thromb Vasc Biol*. 2013;33:2699–2706. doi: 10.1161/ATVBAHA.113.301363.

Significance

This study shows that cholesterol efflux to high-density lipoprotein from interstitial fluid is strongly suppressed in type 2 diabetes mellitus patients. This study analyzes cholesterol efflux in interstitial fluid, an important measurement because the first step in reverse cholesterol transport, namely loading of high-density lipoprotein with cholesterol, occurs in the interstitial compartment. The results contribute important knowledge to understand why type 2 diabetes mellitus patients are at increased risk of developing atherosclerosis.

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Supplementary Table I. Retention time for lipoproteins from serum and IF from T2D patients and healthy controls.

	Serum (minutes)	P value (healthy control vs. T2D patients)	IF (minutes)	P value (healthy control vs. T2D patients)	P value (Serum vs. IF)
Healthy controls					
LDL	39.29±0.31		39.32±0.36		n.s.
HDL	50.42±0.32		50.06±0.42		<0.01
T2D patients					
LDL	39.82±0.41	<0.001	39.68±0.34	<0.001	n.s.
HDL	50.93±0.37	<0.001	50.45±0.60	<0.001	<0.001

Data are means±SD. P values indicate the significance of difference from the same fluid in the control group or between serum and IF within the individual groups, as indicated (Two-way ANOVA followed by Bonferroni's multiple comparison test).

Supplementary Table II. Serum and IF apoAI, and lipid content of HDL from serum and IF, from T2D patients and controls.

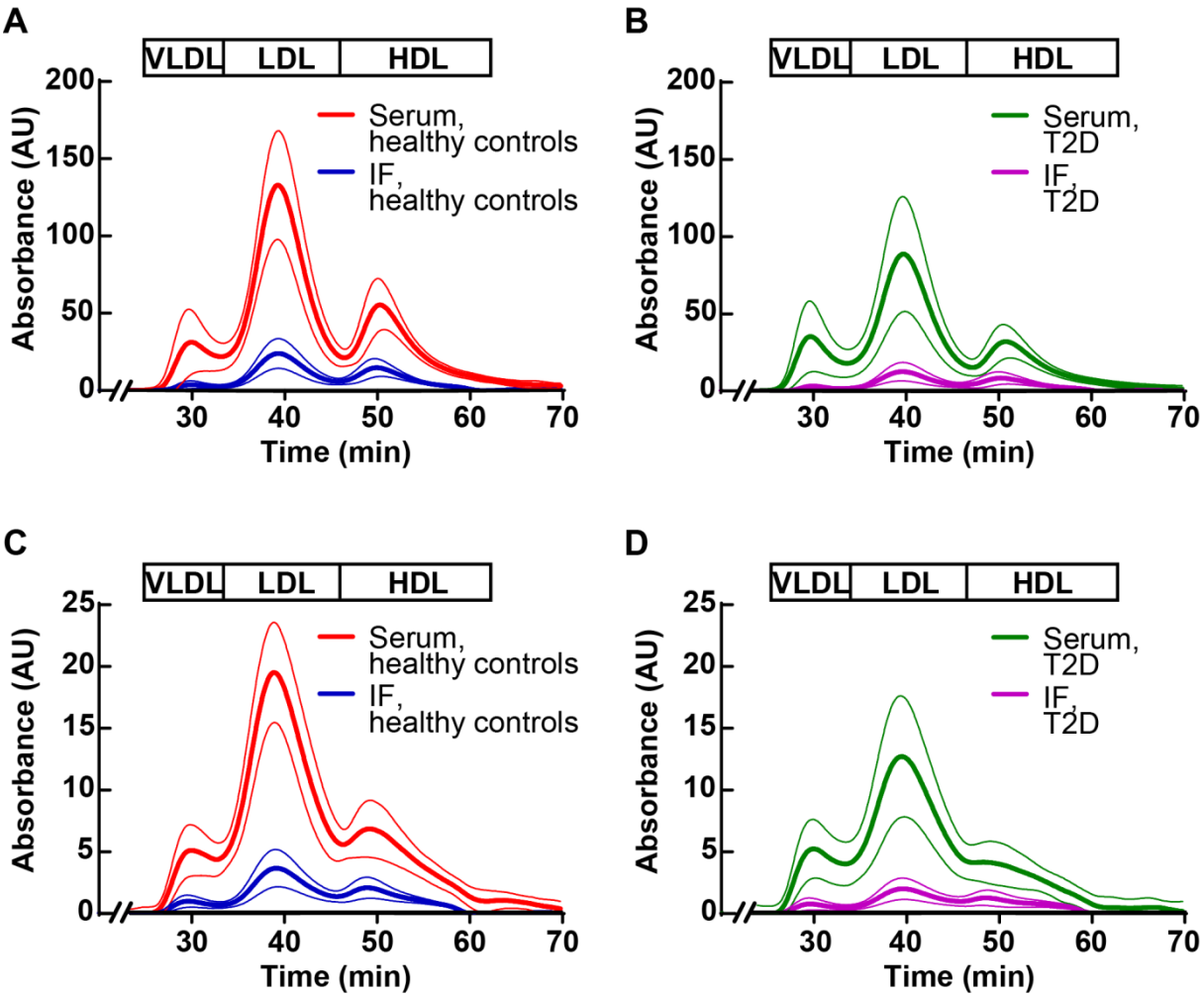
	Serum	P value (healthy control vs. T2D patients)	IF (mmol/L)	P value (healthy control vs. T2D patients)	P value (Serum vs. IF)
Healthy controls					
HDL CE (mmol/L)	1.10±0.32		0.284±0.098		<0.0001
HDL FC (mmol/L)	0.41±0.16		0.123±0.050		<0.0001
HDL TG (mmol/L)	0.183±0.059		0.055±0.019		<0.0001
HDL PL (mmol/L)	2.24±0.57		0.210±0.041		<0.0001
ApoAI (g/L)	0.83±0.14		0.188±0.055		
T2D patients					
HDL CE (mmol/L)	0.67±0.20	<0.0001	0.177±0.081	<0.0001	<0.0001
HDL FC (mmol/L)	0.23±0.12	<0.0001	0.082±0.036	<0.001	<0.0001
HDL TG (mmol/L)	0.167±0.048	n.s.	0.045±0.016	<0.01	<0.0001
HDL PL (mmol/L)	1.47±0.57	<0.0001	0.177±0.040	<0.05	<0.0001
ApoAI (g/L)	0.67±0.14	<0.001	0.124±0.035	<0.0001	<0.0001

CE, esterified cholesterol; FC, unesterified cholesterol. HDL total cholesterol, FC, TG and apoAI data have been published previously (Apro *et al.* J Lipid Res. 2015 Aug;56(8):1633-9) and CE was calculated from total cholesterol and FC concentrations. Data are means±SD. P values indicate the significance of difference from the same fluid in the control group, as indicated (Two-way ANOVA followed by Bonferroni's multiple comparison test). In serum, values for FC (and consequently CE) are missing from three healthy controls, and in IF from one healthy control. One value for serum apoAI in healthy controls is excluded because of being an outlier.

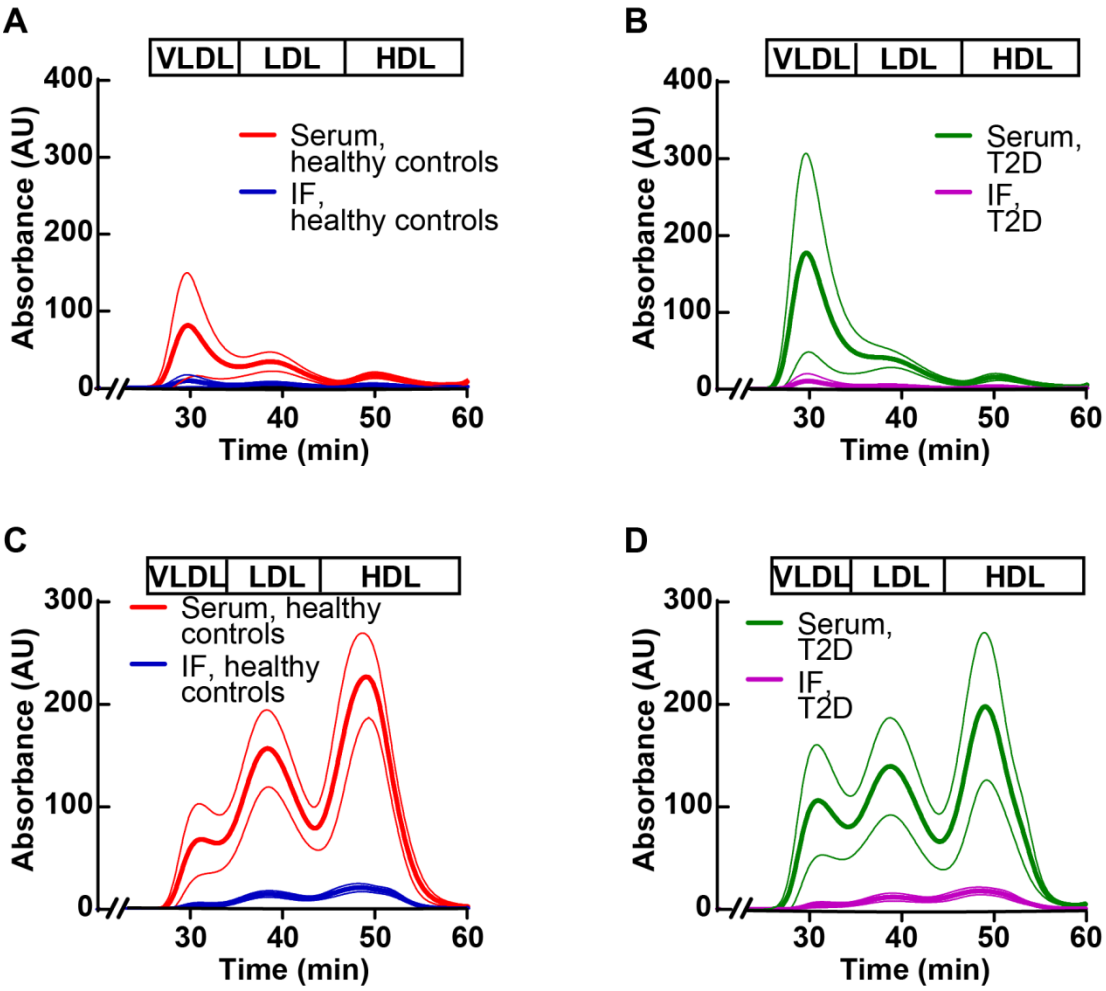
Supplementary Table III. Cholesterol efflux to HDL from plasma and IF from T2D patients treated or not treated with statins and/or fibrates.

	Plasma HDL (%)	P value (Not treated vs. treated with statins/fibrates)	IF HDL (%)	P value (Not treated vs. treated with statins/fibrates)	P value (Plasma vs. IF)
Not treated with statins/fibrates	2.56±0.39		1.71±0.30		<0.0001
Treated with statins/fibrates	2.77±0.35	n.s.	1.88±0.36	n.s.	<0.0001

Data are means±SD. For T2D patients not treated with statins and/or fibrates, plasma was available from 11 patients and IF was available from 7 patients. For T2D patients treated with statins and/or fibrates, plasma was available from 24 patients and IF was available from 17 patients. P values indicate the significance of difference from the same fluid in the T2D group not treated with statins and/or fibrates or between plasma and IF within the individual groups, as indicated (Two-way ANOVA followed by Bonferroni's multiple comparison test).



Supplementary Figure I. FPLC lipoprotein profiles for total (A-B) and unesterified (C-D) cholesterol in serum and IF from healthy controls and T2D patients. Data are presented as means (thick lines) ±SD (thin lines). The approximate elution profiles of the lipoprotein classes are indicated. The absorbance values for IF were divided by five to adjust for the higher sample volume analyzed compared with serum. Data for unesterified cholesterol in serum and IF are missing from three and one of the healthy controls, respectively.



Supplementary Figure II. FPLC lipoprotein profiles for TG (A-B) and PL (C-D) in serum and IF from healthy controls and T2D patients. Data are presented as means (thick lines) ±SD (thin lines). The approximate elution profiles of the lipoprotein classes are indicated. The absorbance values for IF were divided by ten to adjust for the higher sample volume analyzed compared with serum.

Materials and Methods

Subjects and study design
Thirty-five T2D patients and thirty-five age- and gender-matched healthy controls were studied, as described previously.[1] Written informed consent was obtained at inclusion. The study was approved by the regional ethics review board in Stockholm and conducted in accordance with the Declaration of Helsinki.

Collection of IF, serum, and plasma
Peripheral blood samples and suction blister fluid were collected after an over-night fast, as previously described.[1]

Determination of lipoprotein size
Lipoprotein cholesterol profiles were determined with FPLC, as described,[1] and the retention times for the LDL and HDL peaks were used to calculate particle size. For this purpose, a linear regression equation was generated using the mean retention times for serum HDL and LDL in healthy controls (being 50.42 and 39.29 minutes, respectively), together with published particle sizes in healthy subjects (LDL 21.1 nm, and HDL 8.9 nm).[2, 3]

Determination of lipoprotein lipid and apolipoprotein composition
Total and unesterified cholesterol as well as TG were determined as described.[1] PLs were determined in 2 µL of serum and 20 µL of IF, using reagents from WAKO Diagnostics, Richmond, VA. ELISA was used to analyze apoAI (Mabtech, Nacka strand, Sweden), as described.[1]

Assessment of cholesterol efflux
HDL was isolated from IF and plasma by ultracentrifugation (1.063<d<1.21 g/mL) using a Beckman optima TLX ultracentrifuge (Beckman Coulter, Pasadena, CA), followed by dialysis in GeBAflex tubes (Gene Bio-Application Ltd, Yavne, Israel). Cholesterol efflux to HDL was assayed in 48-well plates using THP-1 human monocytes (ATCC via LGC Promochem, Teddington, UK) differentiated into macrophages, essentially as described.[4] Differentiated cells were loaded for 24 hours using RPMI 1640 medium supplemented with 50 µg/mL acetylated LDL and 1 µCi/mL ^{3}H-cholesterol (Perkin Elmer, Boston, MA) followed by equilibration for 24 hours in RPMI 1640 medium containing 2% bovine serum albumin. Efflux was initiated by adding HDL at a final concentration of 1 mg/dL cholesterol. After 5 hours incubation at 37°C, the medium was collected and centrifuged to remove cellular debris. The cells were incubated for at least 30 minutes at room temperature with 0.1 mol/L NaOH. Radioactivity was determined both in medium and cells by liquid scintillation counting (Packard 1600CA Tri-Carb, Packard, Meriden, CT). Efflux per well is expressed as the percentage of counts released into the medium related to the total dose of radioactivity initially present (counts recovered within the medium added to the counts recovered from the cells). Values obtained from control cells without added HDL were subtracted to correct for unspecific efflux. Cholesterol efflux measurements were carried out in all respective samples at the same time to limit potential variation due to different assay conditions. All measurements were performed in duplicate. A standard curve with control HDL was present on each plate to allow correction for potential variations between plates. For efflux analysis, plasma was available from all subjects, while IF was available from 21 healthy controls and 24 T2D patients.

Statistical analysis
Values are presented as mean±standard deviation (SD) and were log transformed prior to statistical analysis performed with two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test (GraphPad Software Inc., La Jolla, CA).

References

1. Apro J, Parini P, Broijersen A, Angelin B, Rudling M. Levels of atherogenic lipoproteins are unexpectedly reduced in interstitial fluid from type 2 diabetes patients. *J Lipid Res*. 2015;56:1633-1639
2. El Harchaoui K, van der Steeg WA, Stroes ES, Kuivenhoven JA, Otvos JD, Wareham NJ, Hutten BA, Kastelein JJ, Khaw KT, Boekholdt SM. Value of low-density lipoprotein particle number and size as predictors of coronary artery disease in apparently healthy men and women: The epic-norfolk prospective population study. *J Am Coll Cardiol*. 2007;49:547-553
3. El Harchaoui K, Arsenault BJ, Franssen R, Despres JP, Hovingh GK, Stroes ES, Otvos JD, Wareham NJ, Kastelein JJ, Khaw KT, Boekholdt SM. High-density lipoprotein particle size and concentration and coronary risk. *Ann Intern Med*. 2009;150:84-93
4. Triolo M, Annema W, de Boer JF, Tietge UJ, Dullaart RP. Simvastatin and bezafibrate increase cholesterol efflux in men with type 2 diabetes. *Eur J Clin Invest*. 2014;44:240-248